The inhibition of the fungus *Botrytis cinerea* by some sesquiterpenoid daucanes

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Daucol 2, 5β, 9α- and 5β, 9β-dihydroxy-(8α-H)-daucane **3** and **5**, have been shown to have a fungistatic action against the plant pathogen *Botrytis cinerea*. The diols were metabolised to 3β, 5β, 9α-trihydroxy-(8α-H)-daucane **7** and 5β, 8α-dihydroxy-(8α-H)-daucan-9-one **8** respectively. These were biologically less active and may represent detoxification products.

Keywords: daucanes, *Botrytis cinerea*, fungistatic action

The fungus, *Botrytis cinerea*, is a serious plant pathogen which attacks economically important crops such as carrots, grapes, lettuces, strawberries and tobacco, producing various leaf spot diseases and a powdery grey mildew.[1] The fungus has developed resistance to some commercial fungicides.[2] In addition some fungicides, such as dicarboximide and procymidone, are persistent enough to be detected in vegetables,[3] soil[4] and in wine even several weeks after vinification.[5] Consequently there is interest in the development of novel rationally designed antifungal agents with activity against *B. cinerea*. We have examined sesquiterpenoid derivatives based on a botryane biosynthetic model[6] in this context. These studies with clovanes have suggested that there may be a key distance between a hydrophobic gem dimethyl group and a hydroxyl group. Molecular models indicate that this relationship may also be found in some daucanes. The purpose of this work was to examine the inhibition of *B. cinerea* by some daucanes with these structural features.

The common sesquiterpenoid daucanes (carotanes),[7] carotol 1[8] and daucol 2[9] have been obtained from carrot seed oil. A daucane which has been isolated[10] from *Gliocladium roseum*, has antifungal activity whilst the simple daucanes also have a formal similarity to known phytoalexins.

Carotol **1** and daucol **2** were isolated from carrot seed oil by distillation and chromatography.[8] Hydroboration of carotol and oxidation of the borane gave 5β, 9α-dihydroxy-(8α-H)-daucane 3. The ^{1}H NMR spectrum of this compound possessed a new C**H**(OH) signal (δ_H=3.30) which was a doublet of double-doublets (*J*=1.8, 9.0 and 10.7 Hz). Irradiation of the 1β-methyl group signal (δ_H=1.01) produced a 9.6% nOe enhancement of the C**H**(OH) signal at δ_H=3.30. Hence the alcohol has the 9α-stereochemistry. Oxidation of the 9-alcohol with chromium trioxide gave a 9-ketone **4** which was reduced with sodium borohydride to give 5β, 9β-dihydroxy-(8α-H)-daucane **5**. The C-9 C**H**(OH) resonance now appeared as a broad doublet (δ_H=3.82, *J*=8.4Hz) in accordance with the hydrogen subtending one large and two smaller dihedral angles with the vicinal hydrogens.

Daucol **2** and the diols **3** and **5** were assayed against *B. cinerea* using a standard agar plate test. The substrate was incorporated into the agar and a 20 mm diameter plug of *B. cinerea* was placed in the centre of the plate. The diameter of the plate which was covered by *B. cinerea* was measured at different time intervals. The results are tabulated (see Table 1). The substrates showed fungistatic activity with 5β, 9α-dihydroxydaucane **3** showing the greater activity. The activity decreased with time suggesting that the compounds were being metabolised to less active products.

Incubation of daucol **2** with *B. cinerea* on surface culture led to a substantial increase in the production of dihydrobotrydial **6**[11,12] (37 mg 1^{-1} *vs* 8 mg 1^{-1} in the control). However, we were unable to isolate any metabolites of daucol from the complex mixture produced by the organism despite growing the fermentation for a number of different time intervals.

One metabolite, **7**, was isolated from the incubation of 5β, 9α-dihydroxy-(8α-H)-daucane **3** with *B. cinerea*. The ^{1}H NMR spectrum of this metabolite possessed three methyl doublets at δ_H=1.14 (*J*=6.7 Hz), 1.22 (*J*=6.7 Hz) and 1.27 (*J*=6.4 Hz).

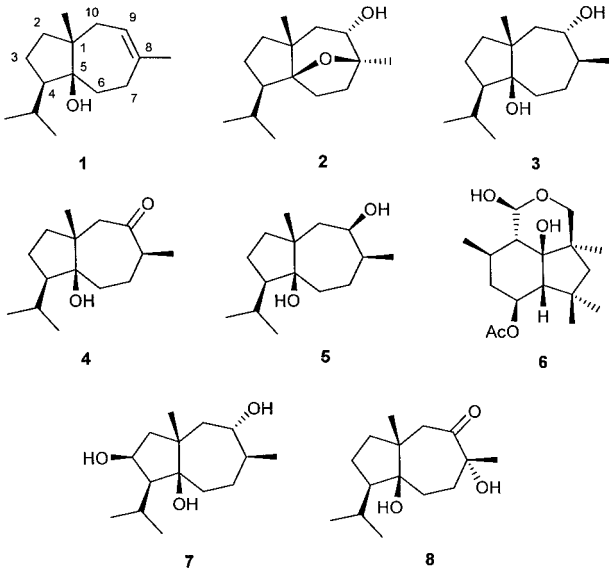

Table 1 Inhibition of *B. cinerea* by daucanes

(% $\frac{\text{decrease in diameter of } B.\ cinerea \text{ substrate}}{\text{diameter of } B.\ cinerea \text{ control}}$ *vs* time)					
Compound	Time				
50 ppm	24	48	72	96	120
2	33	43	30	38	30
3	50	58	22	30	0
5	50	50	9	28	0
7	14	8	12	0	0
100 ppm					
2	33	43	30	49	36
3	83	67	45	64	50
5	66	66	36	42	30
7	28	8	16	4	0
8	28	19	12	28	12
200 ppm					
2	66	71	60	62	40
3	100	92	82	88	80
5	100	92	77	78	56
7	86	85	84	70	46
8	43	44	32	36	18

* Correspondence.

Table 2 ^{13}C NMR data for daucanes

Carbon atom	Compound 3	4	5	7	8
1	46.1	47.3	48.3	47.6	48.3
2	42.9	39.6	41.4	54.1	38.3
3	50.8	52.5	45.1	74.6	45.4
4	58.2	53.3	59.1	60.8	56.0
5	83.0	83.5	82.8	85.0	82.4
6	38.5	35.3	41.0	52.5	31.2
7	27.2	26.1	27.0	39.6	26.1
8	43.7	46.8	42.2	45.3	78.7
9	73.4	213.4	74.2	73.5	214.1
10	25.1	24.9	25.4	27.8	34.3
11	27.0	28.0	29.6	27.9	30.1
12	20.1	15.7	19.7	24.0	21.2
13	21.2	21.7	21.3	24.8	25.3
14	23.7	21.4	23.2	23.4	26.5
15	24.0	23.5	25.1	21.4	24.5

There was also a multiplet at δ_H=3.76 (ddd, *J*=2.5, 9.2 and 11.2 Hz) which was assigned to H-9β. There was a signal for a secondary alcohol (δ_H=4.39, triplet, *J*=3.5 Hz; δ_C=74.5) which was not present in the spectrum of the starting material **3**. The ^1H:^1H 2D COSY spectrum enabled the alcohol to be located at C-3β. The 11-H signal (δ_H= 2.47) was identified since it was coupled to two high-field methyl doublets. The 11-H was also coupled to the 4-H (δ_H=1.3) which was in turn coupled to the new C**H**(OH) resonance. Irradiation of this signal produced nOe enhancements of 5.8% at the 4α-H and 4.3% at the 2α-H. Hence the matabolite has the structure of 3β, 5β, 9α-trihydroxy-(8α-H)-daucane **7**. There was a significant increase in the production of dihydrobotrydial **6** by this fermentation (32 mg l^{-1} *vs* 6 mg l^{-1} in the control).

Incubation of 5β, 9β-dihydroxy-(8α-H)-daucane **5** with *B. cinerea* gave a single metabolite, **8**. The ^1H NMR spectrum of compound **8** possessed two methyl doublets at δ_H=0.82 (*J*=6.6 Hz) and 0.93 (*J*=6.6 Hz) and two methyl singlets at δ_H 0.95 and 1.24 ppm. The 9α-H proton resonance had disappeared and there was a carbonyl signal in the ^{13}C NMR spectrum (δ_C=214.1). A new tertiary alcohol ^{13}C NMR signal appeared (δ_C=78.7) and a methyl doublet in the ^1H NMR spectrum of the starting material had become a singlet (δ_H=1.24). Hence the metabolite contained a C-8 hydroxyl group. The stereochemistry at C-8 was established by nOe experiments. Irradiation of the 1β-methyl group signal (δ_H=0.95) enhanced a double (δ_H=2.24, *J*=11.4 Hz, 2.8%). The same signal was enhanced (3.0%) by irradiating the methyl singlet (δ_H=1.24). Hence the metabolite is 5β, 8α-dihydroxydaucan-9-one **8**. There was a smaller increase in dihydrobotrydial production **6** (10.5 mg l^{-1} *vs* 7 mg l^{-1} in the control). Neither of the metabolites, 3β, 5β, 9α-trihydroxydaucane **7** or 5β, 8α-dihydroxydaucan-9-one **9** showed as much fungistatic activity against *B. cinerea* as the parent diols.

In conclusion we have shown that daucol **2** and 5β, 9α-dihydroxy-(8α-H)-daucane **3** possess fungistatic activity against *B. cinerea* and that there is a lower activity associated with the 9β-epimer **5**. These compounds are metabolised by *B. cinerea* and in the case of the diols, the metabolites, **7** and **8**, have less fungistatic activity and may be detoxification products. The increase in the production of dihydrobotrydial **6** in the presence of the fungistatic agents suggests that this metabolite may be produced by the fungus in some circumstances as a response to stress.

Experimental

General experimental details: Silica for chromatography was Merck 9385. Light petroleum refers to the fraction b.p. 60–80°C. ^1H and ^{13}C NMR spectra were determined at 300 and 75 MHz respectively for solutions in deuteriochloroform. IR spectra were determined as nujol mulls. Mass spectra were determined on a Fisons Autospec mass spectrometer. Extracts were dried over sodium sulfate.

Hydroboration of dauc-8-en-5β-ol (carotol) **1**: The borane: tetrahydrofuran complex (1M, 5 cm^3) was added to a solution of dauc-8-en-5β-ol **1** (310 mg) in dry tetrahydrofuran (30 cm^3) at 0°C under nitrogen. After 4 h water (5 cm^3) was added followed by hydrogen peroxide (27.5%, 4 cm^3) and aqueous sodium hydroxide (10% 4 cm^3). The mixture was left overnight at room temperature. Aqueous sodium sulfite (10% 10 cm^3) and dilute hydrochloric acid (10 cm^3) were added and the mixture was stirred for 30 min. The mixture was extracted with ethyl acetate and the extract was washed with aqueous sodium hydrogen carbonate, water, brine and dried. The solvent was evaporated and the residue chromatographed on silica. Elution with 15% ethyl acetate:light petroleum gave 5β, 9α-dihydroxy-(8α-H)-daucane **3** (210 mg) as an oil, (Found: M^+ 240.2089 $C_{15}H_{28}O_2$ requires 240.2089);ν_{max}/cm^{-1} 3402; δ_H (500 MHz) 0.86(3H, d, *J*=6.7 Hz), 0.91 (3H, d, *J*=6.7 Hz), 0..96 (3H, d, *J*=6.1 Hz), 1.01 (3H, s), 3.30(1H, ddd, *J*=1.8, 9.0, 10.7 Hz).

Oxidation of 5β, 9α-dihydroxy-(8α-H)-daucane **3**: An ice-cold solution of 5β, 9α-dihydroxy-(8α-H)-daucane **3** (155 mg) in acetone (20 cm^3) was treated with the Jones reagent (chromium trioxide in sulfuric acid) (1 cm^3) for 30 min. Methanol was added and the solution was concentrated *in vacuo*. The residue was taken up in ethyl acetate, washed with aqueous sodium hydrogen carbonate, water, brine and dried. The solvent was evaporated and the residue chromatographed on silica. Elution with 10% ethyl acetate:light petroleum gave 5β-hydroxy-(8α-H)-daucan-9-one **4** (126 mg) as an oil, (Found: M^+ 238.1930 $C_{15}H_{26}O_2$ requires 238.1932); ν_{max}/cm^{-1} 3421, 1702; δ_H 0.88 (3H, d, *J*=6.8 Hz), 0.89 (3H, s), 0.95 (3H, d, *J*=6.5 Hz), 0.99 (3H, d, *J*=6.8 Hz), 2.12 (1H, d, *J*=11.5 Hz), 2.66 (1H, d, *J*=11.5 Hz).

Reduction of 5β-hydroxy-(8α-H)-daucan-9-one **4**: An ice cold solution of 5β-hydroxy-(8α-H)-daucan-9-one **4** (120 mg) in methanol (20 cm^3) was treated with sodium borohydride (50 mg) for 30 min. Acetic acid (1.5 cm^3) was added and the solution was concentrated *in vacuo*. The residue was taken up in ethyl acetate, washed with aqueous sodium hydrogen carbonate, water brine and dried. The solvent was evaporated and the residue (114 mg) crystallised from acetone: light petroleum as needles of 5β, 9β-dihydroxy-(8α-H)-daucane **5**, m.p. 66–68°C, (Found: C, 74.8; H, 11.9. $C_{15}H_{28}O_2$ requires C, 74.9; H, 11.7%);ν_{max}/cm^{-1} 3290;δ_H=0.87 (3H, d, *J*=6.6 Hz), 0.92 (3H, d, *J*=6.6 Hz), 0.97 (3H, d, *J*=6.4 Hz), 1.08 (3H, s), 3.82 (1H, br.d, *J*=8.4 Hz).

General fermentation conditions: Botrytis cinerea (UCA 992) was grown on surface culture at 25°C in Roux bottles each containing medium (150 cm^3) comprising (per litre), glucose (40 g), yeast extract (1 g), potassium dihydrogen phosphate (5 g), sodium nitrate (2 g), magnesium sulfate (0.5 g), ironII sulfate (10 mg) and zinc sulfate (5 mg). The substrates, dissolved in ethanol (1 cm^3 per flask), were added 4 d. after inoculation. At the end of the fermentation, the mycelium was filtered, washed with water and ethyl acetate. The broth was acidified to pH 2 and then extracted with ethyl acetate. The combined ethyl acetate extracts were washed with aqueous sodium hydrogen carbonate, dried and the solvent was evaporated to afford the neutral fraction. The sodium hydrogen carbonate extract was acidified and extracted with ethyl acetate. The extract was dried and evaporated but was not further examined. The neutral fractions were chromatographed on silica.

(a) Daucol **2** (250 mg) was incubated with *B. cinerea* (5 1.) for 10 d. Chromatography of the neutral fraction gave the starting material (8 mg) and dihydrobotrydial **6** (185 mg) identified by their ^1H NMR spectra.

(b) 5β, 9α-Dihydroxy-(8α-H)-daucane **3** (250 mg) was incubated for 10 d with *B. cinerea* in 5 1 medium. The neutral fraction from the extract was chromatographed. Elution with 10% ethyl acetate:light petroleum gave the starting material (30 mg). Further elution with 17.5% ethyl acetate:light petroleum gave dihydrobotrydial **6** (161 mg) identified by its ^1H NMR spectrum. Elution with 40% ethyl acetate:light petroleum gave 3β, 5β, 9α-trihydroxy-(8α-H)-daucane **7** (21 mg) which crystallised from ethyl acetate as needles, m.p. 112–114°C, (Found: C, 65.3; H, 10.6. $C_{15}H_{28}O_3$ requires C, 65.6; H, 11.0%);ν_{max}/cm^{-1} 3387; δ_H (500 MHz) 1.14 (3H, d, *J*=6.7 Hz), 1.12 (3H, d, *J*=6.7 Hz), 1.27 (3H, d, *J*=6.4 Hz), 1.53 (3H, s), 3.76 (1H, ddd, *J*=2.5, 9.2 and 11.2 Hz), 4.39 (1H, t, *J*=3.5 Hz).

(c) 5β, 9β-Dihydroxy-(8α-H)-daucane **5** (100 mg) was incubated for 10 d with *B. cinerea* in 2 1 medium. The neutral fraction from the extract was chromatographed. Elution with 10% ethyl acetate:light petroleum gave the starting material (13 mg). Further elution with 17.5% ethyl acetate:light petroleum gave dihydrobotrydial **6** (21 mg) identified by its ^1H NMR spectrum. Elution with 30% ethyl acetate:

light petroleum gave 5β, 8α-dihydroxydaucan-9-one **8** (26 mg) as an oil, (Found: M^+ 254.1880 $C_{15}H_{26}O_3$ requires 254.1881); ν_{max}/cm^{-1} 3446, 1698; δ_H (500 MHz) 0.82 (3H, d, *J*=6.6 Hz), 0.93 (3H, d, *J*=6.6 Hz), 0.95 (3H, s), 1.24 (3H, s), 2.24 (1H, d, *J*=11.4 Hz), 2.91 (1H, d, *J*=11.4 Hz).

Inhibition of B. cinerea: Malt agar (1 l) was prepared from glucose (20 g), malt extract (20 g), peptone (1 g) and agar (20 g). The compound to be tested was dissolved in ethanol to give the appropriate concentration (see Table 1) and added to each plate (1 cm^3 per plate). The final concentration of each substrate was 50,100 and 200 ppm. The ethanol content was identical in the control. A 20 mm disc of actively growing *B. cinerea* was placed in the centre of each plate. The plates were incubated at 25°C in triplicate and the dimeter of the growing culture was measured (see Table 1).

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